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Award Number: DAMD17-02-1-0437

TITLE: Basis of Persistent Microenvironment Perturbation in Irradiated Human Mammary Epithelial Cells

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REPORT DATE: July 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Annual (18 June 02 - 17 June 03)

4. TITLE AND SUBTITLE
Basis of Persistent Microenvironment Perturbation in Irradiated Human Mammary Epithelial Cells

5. FUNDING NUMBERS
DAMD17-02-1-0437

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and
Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates. All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Our hypothesis is that the disruption of morphogenesis releases constraints on genomic integrity in preneoplastic cells. Thus we predict that colonies arising from irradiated cells will show increased genomic instability due to the lack of microenvironment control rather than as a direct result of DNA damage. In the current IDEA grant, we seek to determine whether such a correlation exists. In addition, to be in a position to manipulate the phenotype in future studies, we wish to determine the underlying basis for the irradiated phenotype. In aim 2, we will determine whether irradiated cells can communicate their phenotype in a fashion similar to the 'bystander effect' that occurs when unirradiated cells respond as if they were irradiated under conditions of heterogeneous radiation exposure. In aim 3, we will begin studies to test an alternative or additional mechanism in which the phenotype is perpetuated by epigenetic modifications leading to altered gene expression. Together these studies will test whether the irradiated HMEC phenotype contributes to radiation-induced genomic instability that is observed in cells generations after radiation exposure.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
7

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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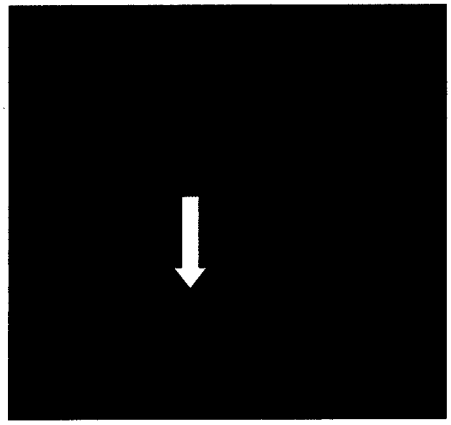
Basis of Persistent Microenvironment Perturbation in Irradiated Human Mammary Epithelial Cells

AIM 1 Determine whether the progeny of irradiated cells have increased indices of genomic instability

Task 1: To determine the frequency of genomic instability in clonal colonies arising from sham-irradiated, TGF-β treatment, irradiated, and irradiated, TGF-β treated cells.

C. Analyze centrosome morphology using immunofluorescence of pericentrin antibodies quantitated with image analysis (Months 1-6).



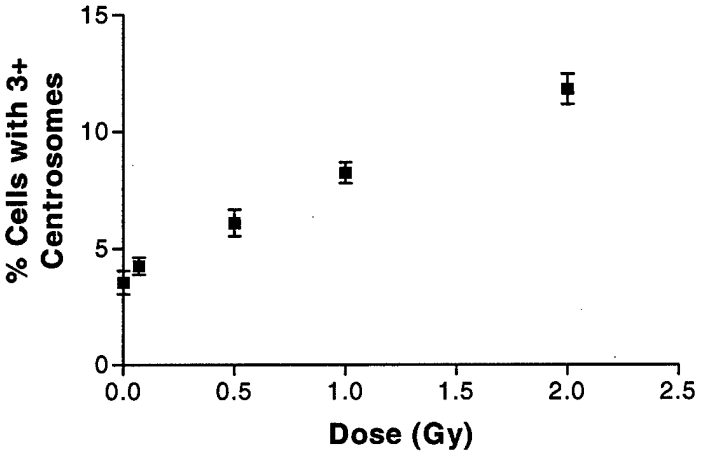


Figure 1: HMT-3522 S1 cells were irradiated to the indicated dose shortly after plating. Surviving cells were fixed 6 days later and stained with using a mouse monoclonal recognizing gamma-tubulin (Sigma) and detected with an Alexa488 conjugated goat anti-mouse secondary antibody (green) and the DNA stained with DAPI (blue). Abnormal numbers of centrosomes (i.e. 3 or more, arrow) were counted manually. Centrosome number per cell was obtained from 8 fields at 40x magnification. The data shown are representative of three independent experiments.

We analyzed centrosome status in the S1 cells as a function of radiation dose. Preliminary studies were carried out on cells grown on tissue culture plastic rather than embedded in rBM. To do so, S1 HMEC were seeded onto chamber slides. On day 0, the cells were irradiated within 4 hours of plating using ^{60}Co γ-radiation and harvested at day 6 for staining with γ-tubulin. After immunofluorescent images were acquired the centrosome status of the cells was determined by manual counting. Cells were indexed as having 1, 2, or ≥3 centrosomes. Although we were unable to optimize the conditions for visualizing centrosomes using antibodies recognizing pericentrin, we were successful with antibodies that recognize another centrosome component, γ-tubulin. Three independent experiments demonstrate a dose dependent increase in the number of cells containing 3 or more centrosomes (Figure 1). These data are consistent with radiation-induced instability in the S1 cells. Preliminary studies show that when cells are grown in the presence of the presence of TGF-β the number of cells containing 3 or more centrosomes is reduced, which suggests that TGF-β protects S1 cells from radiation induced genomic instability. Further studies will be carried out to confirm the TGF-β results.

We have preliminary results, which will be discussed in more detail in later in this report, indicating that TGF-β signaling is increased in irradiated cells. We hypothesize that this increased TGF-β activity is protecting the irradiated cells from increased genomic instability and if we block the TGF-β activity using TGF-β neutralizing antibodies then the number of cells containing 3+ centrosomes would further increase. Experiments to test this hypothesis will be carried out in the near future. These results are exciting since there is little data about the regulation or distribution of centrosomes as a function of TGF-β, or the effect of radiation on centrosomes in non-malignant HMECs.

Dr. Bahram Parvin at LBNL is defining conditions for automated quantitation of the centrosome number, size and morphology. This will expedite analysis of our experiments, as well as facilitate our ability evaluate changes in centrosome size and distribution after irradiation and/or treatment with TGF-β. The work described above was carried out by Rishi Gupta, an undergraduate from the University of California at Berkeley, under the direct supervision of Anna C. Erickson, a postdoctoral fellow funded by DOD-BCRP training grant (P.I.: M.J. Bissell, LBNL). Mr. Gupta's laboratory experience enabled him to obtain an honors degree in Biology. He has recently joined us as a full-time member of the lab and will continue studying the indices of genomic instability in the irradiated human mammary epithelial cells.

Task 2: If the frequency of genomically unstable clones is increased in any of the treated colonies compared to the sham-treatment group, then analyze the correlation between genomic instability and specific microenvironment markers (i.e. E-cadherin, integrin, connexin).
These studies have not yet begun.

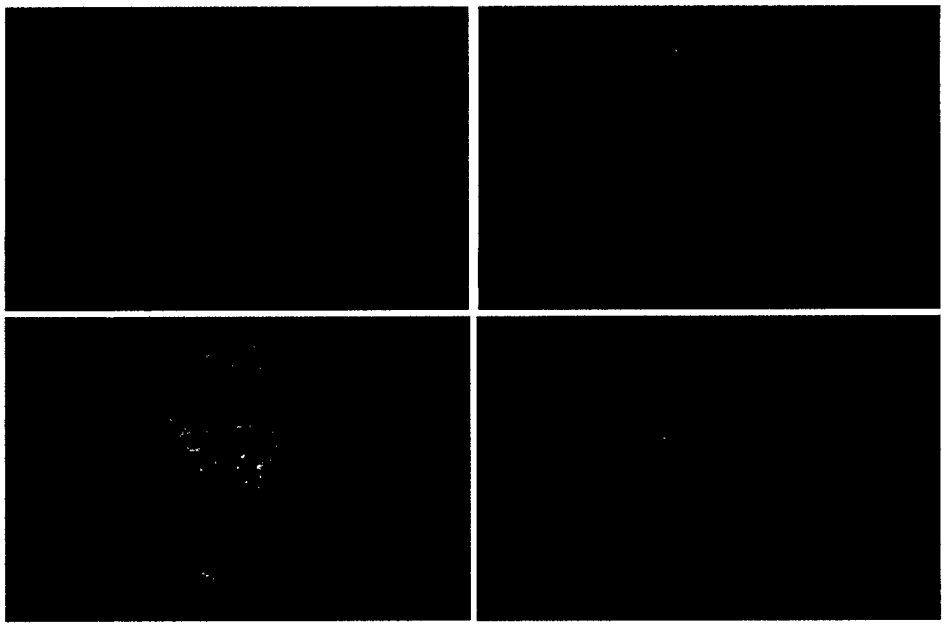
AIM 2 Determine whether the irradiated cells can communicate its phenotype via extracellular signaling.

Task 1: To determine whether co-culture of radiation chimeric populations consisting of differentially marked irradiated and unirradiated cells confer the irradiated phenotype on colonies arising from unirradiated cells.

A. Develop methods and test controls for differential long term labeling of HMEC using Cell-tracker dyes (Months 1-6).

We have determined that labeling the unirradiated cells with Cell-Tracker dyes is preferable since irradiating after labeling causes toxicity. As shown in Figure 2, mixtures of 2:1 irradiated to unirradiated cells form mixed islands. Our initial experiments with these suggest that stabilization of E-cadherin by the cytoskeleton is radically decreased in irradiated, TGF-β treated cells. This phenotype appears to be expressed by the unirradiated cells in mixed culture, suggesting the presence of a soluble or cell-transferred factor.

Figure 2: HMT-3522 S1 cells were dye-labeled (green) before mixing with irradiated (2Gy) cells. 4 days later the cultures were fixed and stained for E-cadherin (red). Note that mixed colonies were obtained.



C. If co-coculture confers the irradiated phenotype, test for the role of soluble factors, such as TGF-β, by exposing un-irradiated cells to the conditioned medium from irradiated cells and by using neutralizing antibodies (Months 18-30).

We found that cell extracts of irradiated monolayer cultures have ~30% less E-cadherin than unirradiated cells by Western blot analysis. In order to investigate if irradiated cells produced a soluble factor which would

result in reduced E-cadherin, we grew unirradiated cells in the presence of conditioned medium (CM) from irradiated cells. Unirradiated cells were grown in CM from unirradiated cells as a control. E-cadherin was reduced in unirradiated cells grown in CM from irradiated cells implying that a soluble factor was involved in the irradiated phenotype.

We know from our other studies and examples in the literature that TGF-β treatment can cause downregulation of E-cadherin in HMECs. In order to investigate the possible role of TGF-β in the downregulation of E-cadherin in the irradiated cells we grew the cells in the presence of TGF-β neutralizing antibodies. The neutralizing antibodies reversed the reduction of E-cadherin in the irradiated cells, suggesting that TGF-β is involved in the irradiated phenotype. With this data in mind and the CM results described above we conducted an additional experiment in which we grew unirradiated cells in the presence of CM from irradiated cells +/- TGF-β neutralizing antibodies. The TGF-β neutralizing antibodies reversed the reduction in E-cadherin protein caused by the irradiated CM. These results suggest that TGF-β is involved in establishment of the irradiated phenotype.

AIM 3 Test the hypothesis that epigenetic molecular mechanisms perpetuate the irradiated phenotype from generation to generation of human mammary epithelial cells.

Task 1: To determine with the loss of E-cadherin immunoreactivity and protein abundance is a function of hypermethylation of the E-cadherin gene in population arising from sham, irradiated, TGF-β treated, or dual treated cells

A. Establish and validate the methylation-specific E-cadherin assay in our laboratory (Months 1-9).

Graff et al. (1997) has mapped the patterns of nine CpG island methylations for the E-cadherin gene in normal and neoplastic cells. To establish conditions for methylation specific PCR (MSP) of the E-cadherin gene genomic DNA was isolated from MDA-MB-231 and MCF-7 cell lines. MDA-MB-231 cells do not express E-cadherin due to silencing of the E-cadherin gene by methylation whereas MCF-7 express E-cadherin. The genomic DNA was subjected to bisulfite modification and PCR amplication was carried out using primer pairs previously described for three separate CpG islands in the E-cadherin gene (Graff et al. 1997). One of the primer pairs (A2) amplifies a region in both cell lines that is methylated while the other two primer pairs (IS2 and IS4) amplify regions which are methylated only in MDA-MB-231 cells. As expected we found that the A2 region was methylated in both cell lines and when MSP was carried out using primers to the IS2 and IS4 regions only the MDA-MB-231 DNA was methylated. We have successfully established this technique in our lab and have both positive and negative controls available when evaluated our sample genomic DNA.

B. Determine whether the E-cadherin gene is hypermethylated in the progeny of treated cells.

Silencing of the E-cadherin gene would result in reduction of the E-cadherin mRNA. Before carrying out MSP we carried out quantitative RT-PCR using a Lightcycler real-time PCR machine (Roche) for the E-cadherin message on RNA isolated from monolayer cultures treated with +/-2Gy and/or +/- TGF-β. Cells treated with TGF-b or TGF-b and 2Gy irradiation showed a marked reduction in E-cadherin message, compared to untreated cells, which correlated with the decreased E-cadherin protein found in these cells. While RNA from 2Gy irradiated cells showed only a slight decrease in E-cadherin mRNA levels compared to untreated cells. Since E-cadherin mRNA levels were altered we followed through with MSP for the E-cadherin gene.

Genomic DNA was recovered from cells grown in monolayers or embedded in rBM which had been treated with +/-2Gy γ-irradiation and/or +/-TGF-β. MSP was carried out using the primers and controls described above. Treatments did not change the methylation patterns for the sites we examined suggesting that methylation of these three sites in E-cadherin promoter is not the mechanism by which E-cadherin protein abundance is reduced in the irradiated phenotype.

C. If so, we will examine the phenotype of treated cells exposed to 5-azacytidine or 5-aza-2'-deoxycytidine (5azadC) to reverse methylation.

We knew from the MSP experiments that the E-cadherin promoter was not differentially methylated between treatment groups. Instead of approaching this experiment with the idea of demethylating the E-cadherin gene and inducing its expression we came from a different point of view and asked if methylation played a role in the irradiated phenotype. MDA-MB-231 cells were used as a control for determining toxicity and efficiency of the 5azadC treatment. It was determined that 2.5μM 5azadC was not toxic to the cells and was sufficient to induce E-cadherin expression.

We carried out preliminary experiments in which TGF-β, irradiated, or dual treated cells were grown in the presence or absence of 2.5μM 5azadC. E-cadherin protein abundance was monitored via Western blot analysis. In the absence of 5azadC the cells displayed the same pattern of E-cadherin abundance that was described previously. There is a reduction in E-cadherin protein after 2Gy irradiation and E-cadherin abundance is decreased further when cells are grown in the presence of TGF-β, +/- 2Gy. When 5azadC was present under the same conditions there was a slight reversal or increase in 2Gy and dual treated cells, but TGF-β cells displayed an additional reduction in E-cadherin abundance. This experiment will have to be repeated in order to confirm these results.